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Metabolic activation of 1,2-dibromoethane to a free radical intermediate by rat liver microsomes and isolated hepatocytes

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A one-electron reductive metabolism of 1,2-dibromoethane (DBE) is described that gives rise to a free radical intermediate, which can be stabilized by a spin trapping agent and detected by electron spin resonance spectroscopy. Using rat liver microsomes or isolated hepatocytes from phenobarbitone pretreated animals, under hypoxic conditions, it has been possible to trap a free radical intermediate and identify it by using ^{13}C-DBE. Inhibition experiments have demonstrated that the site of activation is the microsomal drug metabolizing system.

1,2-Dibromoethane *Free radical* *Spin trapping* *Hypoxia* *Reductive metabolism* *Cytochrome P450*

1. INTRODUCTION

1,2-Dibromoethane (DBE) is a synthetic chemical used primarily in antiknock additives to petrol; other uses include pesticidal fumigation of stored grain, vegetables and fruits, and preplanting treatment of soil to protect against nematodes [1,2].

Reports of the high toxicity of DBE for animals and humans appeared a number of years ago [3–5]. DBE produces respiratory lesions in rats and mice [6,7]; abnormal spermatogenesis in bulls [8,9] and in rams [10]; is carcinogenic in rodents [2,11]; is a mutagen in microorganisms [12,13] and in mammalian cells, both in the absence or in the presence of a metabolizing system [14]. The effects of chronic exposure in man are not certain although abnormal spermatogenesis and several cases of cancer have been reported [15] following industrial exposure.

The metabolism of DBE has been studied both in vivo and in vitro [16–18]. Two main pathways have been suggested: one involves a glutathione transferase-mediated conjugation of DBE with glutathione (GSH), which eventually results in the formation of mercapturic acid derivatives; the other is based on the oxidative dehalogenation by microsomal enzymes, resulting in bromoacetaldehyde. However, by analogy with other halogenated compounds such as CCl_4 and $CBrCl_3$ and halothane [19,20], a cytochrome P450-mediated one-electron reduction of 1,2-dibromoethane can be postulated that will give rise to a free radical intermediate. Such free radicals can often be detected by electron spin resonance (ESR) spectroscopy associated with the spin trapping technique [21], where the free radical interacts with a spin trap possessing a nitrone functional group to form a comparatively long-lived nitroxide radical adduct.

We now report on the existence of a one-electron reductive pathway for DBE, under hypoxic conditions, resulting in the formation of a free radical intermediate and on its positive identification using the spin trapping technique and [^{13}C]DBE.

2. MATERIALS AND METHODS

1,2-DBE (C. Erba, Milano) was redistilled before use; [^{13}C]DBE was obtained from Merk, Sharp and Dohme Limited (Canada); phenyl-*t*-butylnitrone (PBN) was purchased from Aldrich Chemi-

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cal Co. (Milwaukee WI); collagenase (type I) was supplied by Sigma Chemical Co. (Poole, Dorset); other reactants of the highest commercial grade were used without further purification.

Male Wistar rats, maintained on a standard laboratory diet and water 'ad libitum' were used for all the experiments. Phenobarbitone (PB), when required, was administered in drinking water at 1 g/l for 5 days before the experiment.

Rat hepatocyte suspensions were prepared as in [22]. Samples (1.5 ml) of the suspension containing 10^7 cells/ml were incubated in the presence of the spin trapping agent (PBN 25 mM) at 37°C for 15 min in stoppered (50 ml) Erlenmeyer flasks that had central wells. Microsomes were prepared as in [23]. DBE was added in the central well of the Erlenmeyer flasks. Hypoxic conditions were obtained by gentle blowing of oxygen-free nitrogen on the suspension for 10 min before adding DBE.

The oxygen concentration was measured using a Clark-type electrode. Differential spectra were recorded using a DU 8 Beckman spectrophotometer.

A Varian Associates E4 spectrometer, fitted with a variable temperature cavity was used for the ESR analysis as in [20]. Gas–liquid chromatographic analyses were carried out on a Varian 3600 gas–liquid chromatograph, fitted with a flame ionisation detector and using a 80/100 carbopack C/0.1% SP 1000 column (Supelco); detector and injector temp. 200°C; oven temp. 120°C.

3. RESULTS AND DISCUSSION

Aerobic incubation of rat liver hepatocytes in the presence of PBN 25 mM and DBE at 37°C did not give rise to any radical adduct (fig.1b). When the same kind of incubation was performed under hypoxic conditions using hepatocytes from phenobarbitone induced rats, a radical adduct was detected by ESR (fig.1c). The spectrum shows a triplet of doublets, the hyperfine splitting constants were $a_N = 14.5$ G and $a_H = 2.15$ G. The same spectrum was obtained from microsomal suspensions.

These data are not sufficient for the assignment of the above spectrum to the DBE free radical species, therefore [^{13}C]EDB was used instead of [^{12}C]DBE.

The paramagnetic carbon-13 nucleus ($S = 1/2$) gives rise to a splitting of the spectrum lines when

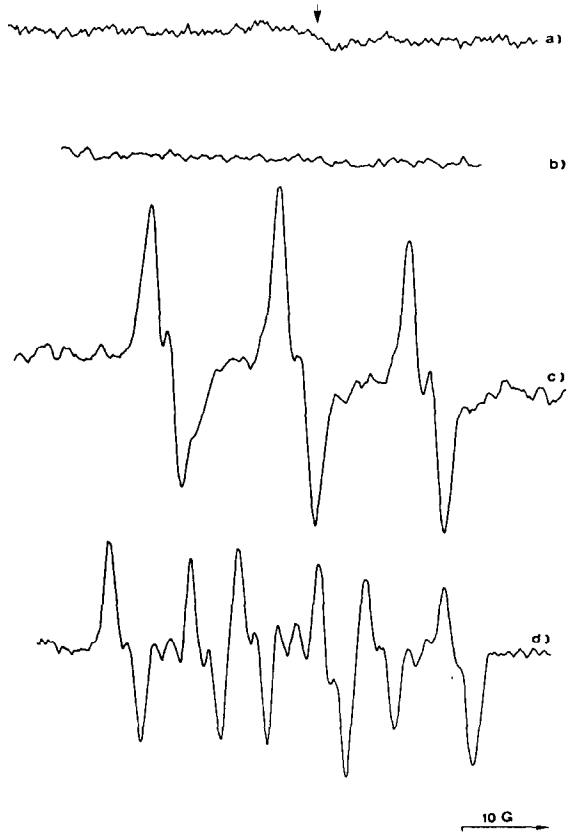

Fig.1. ESR spectra obtained from isolated rat hepatocytes incubated at 37°C for 15 min in the presence of the spin trapping agent PBN. (a) no addition of DBE; (b) hepatocytes in normoxic conditions, with DBE (2.5 μl) added to the central well; (c) hepatocyte suspension under hypoxic conditions, with DBE (2.5 μl) added to the central well; (d) as (c), but after adding [^{13}C]DBE, 90% of ^{13}C, (2.5 μl) to the central well.

it reacts with the spin trapping agent and is located β to the nitroxide group. Fig.1d shows the effect of the introduction of [^{13}C]DBE in the incubation mixture; the characteristic hyperfine splitting, $a_{^{13}C} = 9.2$ G clearly indicates that the radical species trapped is indeed the DBE-derived free radical. The hyperfine splitting effect due to ^{13}C was proportional to the atom % of ^{13}C relative to ^{12}C in the DBE added to the system. Oxygen was $<20\,\mu$M at the beginning of the incubation time and was reduced below 1 μM after about 5 min incubation.

The effects on the signal intensity by varying the amount of DBE added to hepatocytes and microsomes suspension are shown in fig.2. In both the

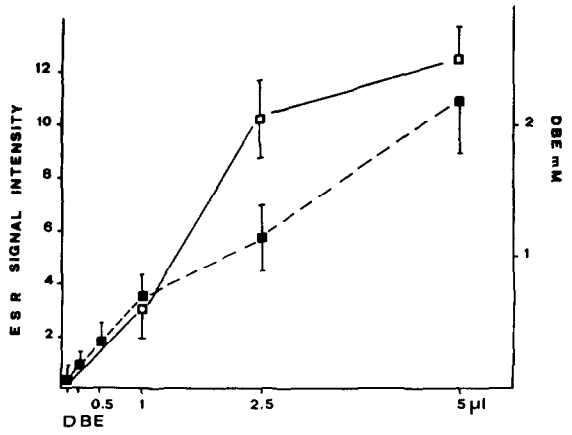

Fig.2. (■) Concentration of DBE in the hepatocyte suspension increases with the amount of DBE added to the central well. (□) Intensity of the ESR signal (arbitrary units) increases the increase of DBE added to the central well. Mean values ±SEM are shown.

experimental systems the intensity of the DBE-derived adduct is directly proportional to the amount of DBE added.

Hepatocytes and microsomes prepared from rats that had not been induced with PB or from 3-methylcholantrene-pretreated rats did not give any appreciable radical adduct formation.

To determine the final concentration of DBE in the incubation mixture, gas–liquid chromatographic analysis has been carried out on the suspensions. The use of Carbopack C allowed the direct introduction of the cell suspension into the column, without any previous distillation or extraction in organic solvent. Fig.2 shows the linear relationship between the increase of DBE added in the central well and the rise in DBE concentration found at 10 min incubation.

Double beam differential spectroscopy [24] of DBE was carried out on phenobarbitone-pretreated rat microsomes and showed type I spectra, both under aerobic and anaerobic conditions, with a trough at 420 nm and a peak at 391 nm.

Experiments with cytochrome P450 inhibitors such as SKF 525A, metyrapone, *p*-chloromercuribenzoate (*p*-CMB) and carbon monoxide demonstrated a decrease in the radical adduct formation, carbon monoxide being the most effective (fig.3). The stimulation produced by pretreatment with phenobarbitone, the formation of type I spectra, and the effect of the inhibitors imply the involvement of the cytochrome P450 in the radical activation of DBE; the drug metabolizing system performing a one-electron reduction of the compound and a consequent dehalogenation with the formation of the free radical $H_2CBr–H_2C^{\cdot}$ or $H_3C–HCBr^{\cdot}$). The low oxygen concentration is decisive for a significant reductive type of metabolism. With similar conditions a reductive dehalogenation mechanism involving a radical–ferric cytochrome complex has been reported for chlorinated alkanes [25].

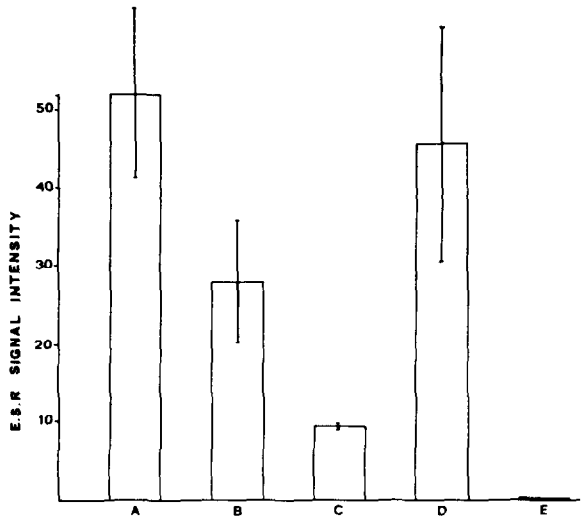

Fig.3. Effects of various inhibitors of the microsomal drug metabolizing system on the formation of DBE-derived free radical with PBN arbitrary units. For each inhibitor the number of experiments was = 4. The standard error of the mean has been reported on each bar of the plot. (A) Microsomes incubated with DBE (2.5 µl in the central well); (B*) addition of methyrapone (0.5 mM); (C°) addition of SKF 525 A (0,5 mM); (D) addition of *p*-chloromercuribenzoate; (E°) addition of carbon monoxide (1 min flow); (*) significatively different from A ($p < 0.05$); (°) significatively different from A ($p < 0.005$).

Taking into account that, under physiological conditions, the average hepatic oxygen concentration is about 35 µM, and that the presence of an intrahepatic oxygen gradient is well established, a reductive type of metabolic activation of DBE may be possible even with a normal blood oxygen supply to the liver in vivo [26,27].

These data clearly demonstrate the presence of a new metabolic pathway for DBE. Further studies

are needed for the determination of the reactivity of the radical intermediate and its relevance to the toxicity and carcinogenicity of the parent molecule.

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